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Relationship between Actinic Damage and Chronologic Aging in Keratinocyte Cultures of Human Skin

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The relationship of actinically-induced "premature aging" to chronological aging was studied in paired keratinocyte cultures obtained from the habitually sun-exposed (lateral) and nonexposed (medial) aspects of the arm of 5 male donors, aged 41 to 80 yr. In all cases, the number of cell generations *in vitro* was greater for cultures derived from non-exposed skin than for cultures derived from sun-exposed skin, and this discrepancy increased with donor age and the severity of clinical aging changes. Hence, chronic sun exposure does accelerate aging in human skin by at least one previously established *in vitro* criterion: it decreases the lifespan of cultured keratinocytes. Plating efficiency was 11- to 32-fold higher for keratinocytes from chronically sun-exposed skin than for nonexposed controls, perhaps reflecting the recognized carcinogenic potential of actinic radiation. Keratinocyte cultures appear to be as amenable to gerontologic studies as the already widely used human fibroblast cultures.

Clinically recognized age-associated changes in skin are wrinkling, loss of elasticity, mottled pigmentation, vascular ectasia, atrophy, and benign proliferative growths such as seborrheic keratoses, acrochordons and cherry angiomata [1-3]. These changes are considerably more prominent in chronically sun-exposed areas and in fair-skinned individuals relatively lacking a melanin barrier. "Premature aging" due to sun exposure is now widely accepted by dermatologists as well as by the lay public. The possible significance of this phenomenon is more than cosmetic, however, for if actinic radiation can indeed be shown to accelerate aging in skin, it will be the first environmental factor demonstrated to affect the aging process.

Formation of stratified epidermal colonies *in vitro* from single cells is possible utilizing the technique of Rheinwald and Green [4,5], in which a lethally irradiated fibroblast monolayer supports epidermal proliferation and differentiation. This system provides an opportunity to increase our understanding of the aging process in human skin by complementing existing data for dermal fibroblasts with comparable data for a differentiating cell type, the keratinocyte. The following study was performed to determine whether sun-exposed clinically aged skin is indeed "older" than nonexposed skin of the same individual, according to accepted criteria for aging *in vitro* [6,7].

MATERIALS AND METHODS

Patient Material

Eight adult male Caucasian volunteers aged 41 to 80 yr, known to be in good general health and specifically not to have diabetes, were recruited for this study. All were moderately fair-skinned life-long residents of New England with similar histories of outdoor activities. No subject was using any medication except multivitamins. Biopsies were performed during the winter and early spring months.

Four millimeter diameter punch biopsies, using local 2% xylocaine anesthesia, were obtained from normal-appearing skin of the medial (nonexposed) and lateral (sun-exposed) aspects of the upper arm. Informed consent was obtained from all subjects.

Culture Techniques

Minor modifications of the procedures described by Rheinwald and Green were used [4,5].

Primary Cultures

Biopsy specimens were placed immediately in sterile normal saline, coded by a third party, and returned to the investigator, who subsequently did not know which biopsy was chronically sun-exposed and which nonexposed. In the laboratory, subcutaneous fat and deep dermis were dissected away with scissors. The remaining tissue was minced and placed in 10 cc of .25% trypsin and .01% EDTA in a stirring flask at 37°C for 45 min. After allowing the minced biopsy material to settle, the supernatant, containing single cells, was withdrawn and replaced with fresh trypsin and EDTA solution. The supernatant was centrifuged for 6 min at 600 rpm, resuspended, counted in a hemocytometer and plated at densities ranging from 10^4 to 10^5 cells/60 mm dish (approx. 3 to 30 cells/mm^2) in Falcon tissue culture dishes on a monolayer of 3T3 fibroblasts which had been lethally irradiated and plated the preceding day. In most cases, 2 trypsinization cycles were used to secure a total of 10^5 to 10^6 cells. After these 2 cycles, distinct epidermal fragments could no longer be recognized in the trypsinization flask. The entire procedure, from biopsy to final innoculation of plates, never exceeded 3 hr. All cultures were maintained in Dulbecco's modified Eagle's medium with 20% fetal calf serum, hydrocortisone 0.4 ng/ml, penicillin G 75 units/ml, and streptomycin 50 ng/ml. Medium was changed 5 days after innoculation and twice weekly thereafter.

Feeder Layer

3T3 mouse teratomal fibroblasts were obtained from Dr. Howard Green, Department of Biology, Massachusetts Institute of Technology, and serially cultivated in Dulbecco's MEM with 10% calf serum, penicillin and streptomycin. Medium was changed twice weekly. Prior to use as a feeder layer for epidermal colonies, 3T3 cells were plated at 5 $\times$ 10^5 cells/60 mm dish and irradiated with 6000 R over 5 min in a cobalt source.

Subcultures

Plates with established epidermal colonies were sprayed vigorously with .02% EDTA (which dislodges 3T3 and contaminating human fibroblasts but not keratinocytes), rinsed, incubated in .25% trypsin for 15 min at 37°C and then checked under the microscope at 5 min intervals until no adherent keratinocytes could be seen. Trypsin was neutralized with complete medium and the resulting single cell suspension was centrifuged, resuspended, counted, and replated on a fresh 3T3 monolayer.

Staining

Cultures were fixed in 10% formalin, stained for 30 min with Rhodanile blue (equal volumes of 2% Rhodamine B and 2% Nile Blue), and then rinsed with water for 1-2 min to remove excess stain.

Definitions

Plating efficiency: the number of epidermal colonies visible on a stained plate after 3 weeks growth, divided by the total number of cells originally plated.

Average number of cells per colony (CPC): After removing fibroblasts

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Abbreviation:
CPC: cells per colony

from the plate, the remaining cells (keratinocytes) are trypsinized and counted, and this number is divided by the number of colonies counted on a paired stained plate.

Number of keratinocyte generations per passage (G): 2^G = number of cells created by a single progenitor after G generations, i.e., the number of CPC. $G = \frac{\ln (CPC)}{\ln 2}$. At the plating density employed for these determinations (10^4 to 2×10^4 cells/60 mm dish) it is safe to assume that all colonies arise from single cells. This calculation also assumes that every cell capable of establishing a visible colony of at least 8 cells (i.e., of dividing at least 3 times) and all its progeny subsequently continue to divide at the same rate as all other colony-forming cells on the plate.

In the last passage, colonies were frequently abortive, consisting of highly keratinized aggregates with fewer than 8 cells visible on stained plates. In this situation, CPC and G were estimated to be <8 and <3 respectively without attempting to count total keratinocytes from paired plates.

RESULTS

In every instance, there were striking clinical differences between the chronically sun-exposed and nonexposed aspects of the upper arm. Skin on the outer aspect of the arm was darker and "thicker," or more difficult to pinch between two fingers; and had more telangiectasia, irregular hyperpigmentation, wrinkling, and benign epidermal proliferations (seborrheic keratoses). Skin on the inner aspect of the arm was pale and smooth in all subjects. In older subjects, there was "sagging" and loss of elasticity, while in subjects less than 60 yr old, the skin usually appeared completely normal. No patient had erythema or a dark tan to suggest recent sun exposure; and no patient had lesions on the arm compatible with actinic keratoses, basal cell carcinoma or squamous cell carcinoma. Histologic sections confirmed these clinical observations (Fig 1).

Biopsy material from three subjects was bacterially contaminated and had to be discarded within the first week of culture. The remaining 5 sets of cultures all yielded stratified epidermal colonies which were first visible at the 8 to 32 cell stage, 8 to 12 days after initial plating. Most colonies grew in an orderly manner over the subsequent 2 to 3 weeks, remaining circular in configuration with tightly adherent cells (Fig 2).

On most plates there was a wide range of colony size, so that after 3 weeks colonies containing more than 1000 cells were intermixed with abortive colonies of fewer than 50 cells, as well as colonies of intermediate size. All cultures obtained from a single skin specimen were similar in plating efficiency and distribution of colony sizes, however.

Four of the 5 subjects formed visible colonies in secondary cultures; tertiary cultures were achieved in only one instance. Vigorous growth was restricted to the primary cultures in the case of nonexposed skin in 4 of the 5 subjects and, in the case of exposed skin, in all 5 subjects. The plating efficiency for primary cultures obtained from biopsies of nonexposed skin was approximately .01% in all cases.

In each of the 5 subjects, proliferative capacity was greater among cells cultured from chronically sun-exposed skin (E cells) than among cells cultured from nonexposed skin (non-E cells) (Table). The plating efficiency of E cells was 11- to 32.5-fold higher than that of non-E cells (Fig 3), and the total keratino-

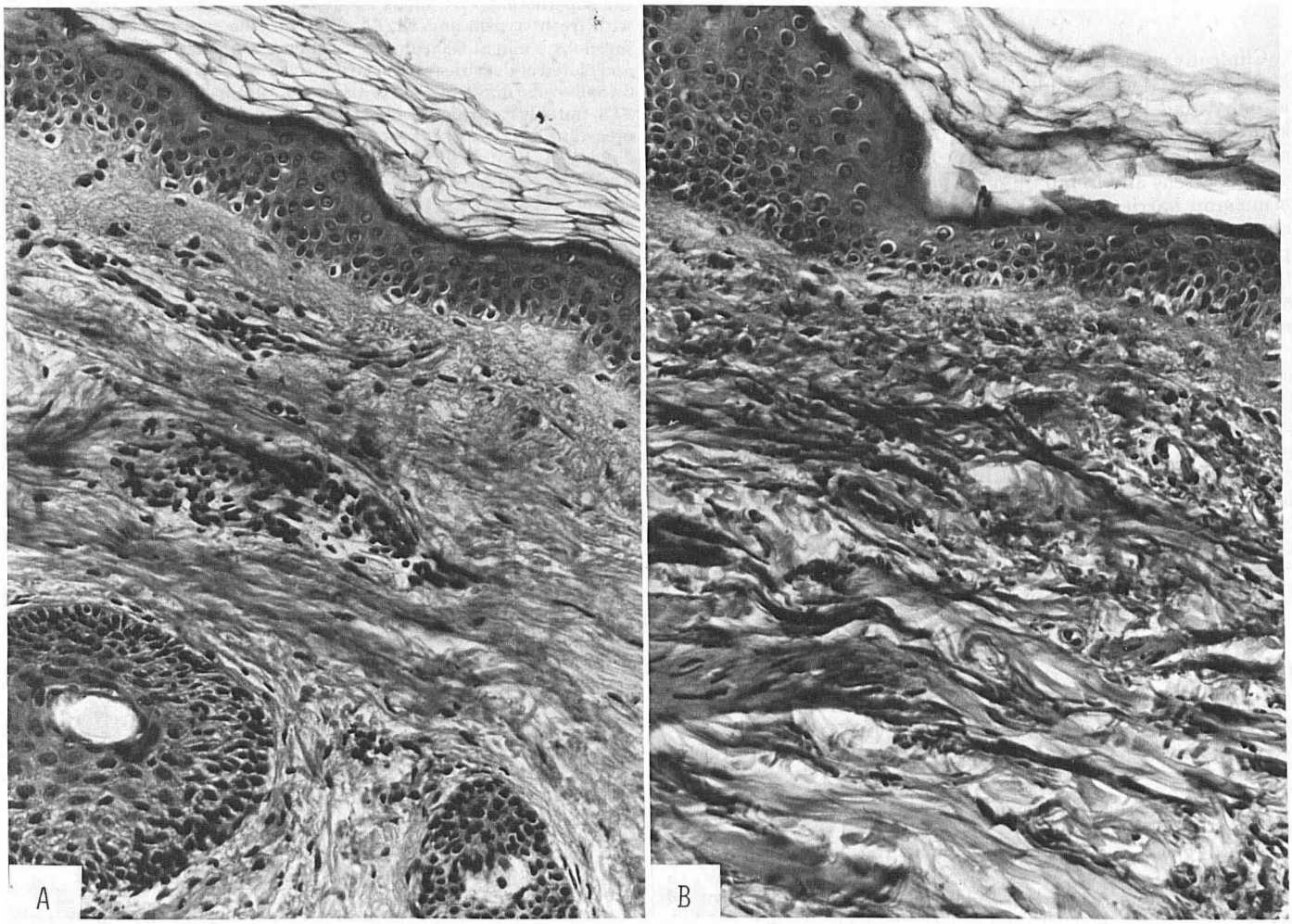

FIG 1. Histologic sections of skin biopsies obtained from subject 2. *A*. Nonexposed, medial aspect of the arm. Note uniform thickness of the epidermis and normal-appearing dermal collagen. *B*. Sun-exposed, lateral aspect of the arm. Note focal hyperplasia of the epidermis, lack of atypia, and moderate solar elastosis (Hematoxylin and eosin, reduced from × 200).

cyte yield per plate was also greater. However, the average colony size achieved during the first passage and number of cell generations was always greater for the non-E cell cultures, with non-E cell colonies 40% to 10.5 fold larger than E cell colonies on the average.

The discrepancy between both colony size and number of generations for sun-exposed vs nonexposed keratinocytes varied with donor age. The youngest subject (41 yr old) had only a 6% difference between number of generations in his paired cultures, while the oldest subject (80 yr old) had a 45% difference; the other 3 subjects had intermediate values (Fig 4). No significant effect of donor age on culture lifespan could be detected in this small group.

DISCUSSION

Current concepts of the aging process are based largely on studies of human diploid fibroblasts. The pioneering work of Hayflick and Moorehead established that human fetal fibroblasts have a finite *in vitro* lifespan of approximately 50 generations [8,9], and others have subsequently shown that the lifespan of fibroblasts obtained from human skin biopsies is inversely proportional to the donor's age [7,10] and decreased in individuals with progeria [7,11,12], Werner's syndrome [7], and diabetes [7,13,14], all conditions considered by some authors to constitute "premature aging." Plating efficiency or the percentage of cells capable of division has also been shown to decrease as a function of *in vitro* age for human diploid fibroblasts [15].

In a small number of biopsy specimens, Rheinwald and Green found a similar range and rate of decline in culture lifespan for epidermal cells with increasing donor age [5]; 7 neonatal foreskin cultures achieved from 25 to 51 generations, while 1 skin biopsy each from 3, 12 and 34 yr old donors yielded only 26.5,

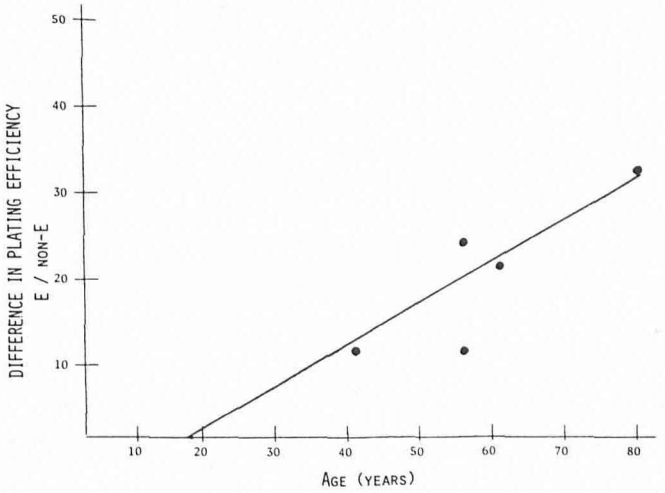

FIG 3. Differences in plating efficiency for primary cultures of keratinocytes from chronically sun-exposed (*E*) and nonexposed (*non-E*) sites, E/non-E, as a function of donor age. Note 11- to 32.5-*fold* difference. Coordinates obtained from the Table and regression line by the method of least squares. (y = .5x − 9, correlation coefficient = .83).

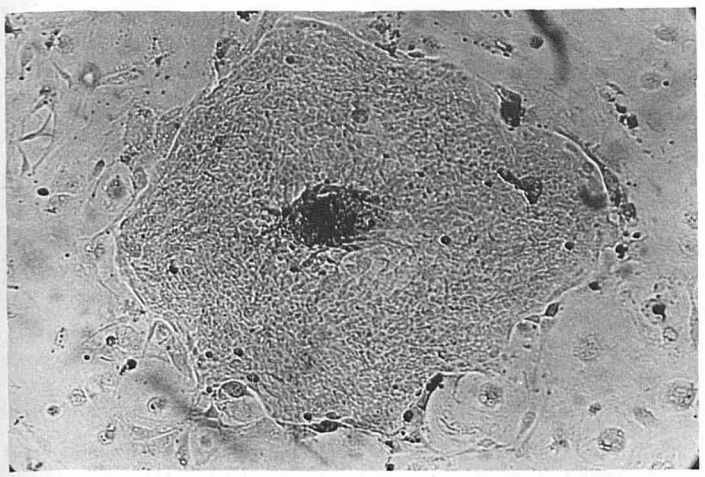

FIG 2. Typical keratinocyte colony, 21 days after initial plating. Note tightly aggregated cells, overall circular configuration of colony, and dark central keratinization. Large sparse cells at the periphery are the 3T3 fibroblast feeder layer (reduced from × 150).

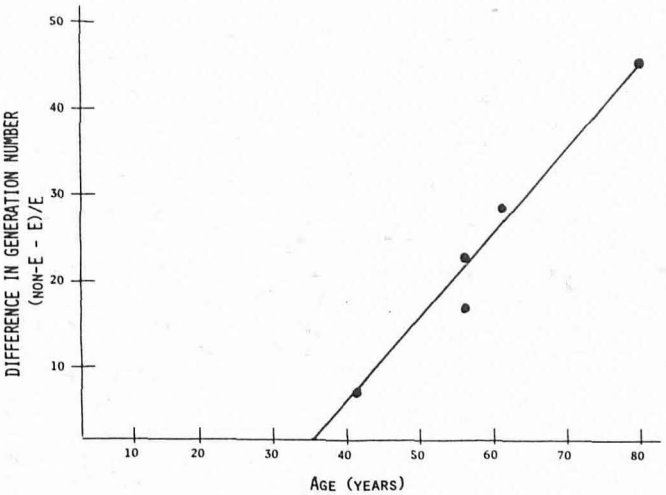

FIG 4. Discrepancy in the average number of generations achieved in culture by keratinocytes from chronically sun-exposed (*E*) and nonexposed (*non-E*) sites, (*non E* − *E*)/E, as a function of donor age. Coordinates obtained from the table and regression line by the method of least squares. (y = x − 35, correlation coefficient = .99).

Culture Characteristics of Human Epidermal Cells from Adult Donors

Subject	Age (yr)	Passage No.	Plating efficiency × 10^{-2}		Cells per colony[a]		Generations	
			Non-E[b]	E[c]	Non-E	E	Non-E	E
1	61	1	.008	.17	7550	1100	12.9	10.1
		2	.10	.56			<3	<3
2	56	1	.010	.11	3550	800	11.8	9.6
		2		.016				<3
3	80	1	.008	.26	2050	200	11.0	7.6
		2	.14	.04	450		8.8	<3
		3	.06				<3	
4	56	1	.009	.22	1350	500	10.4	8.9
		2		.15				<3
5	41	1	.010	.14	650	450	9.3	8.8

[a] Numbers rounded to nearest 50.
[b] Non-E: nonexposed aspect of arm.
[c] E: sun-exposed aspect of arm.

23, and 20 generations respectively. Plating efficiencies in this study ranged from .15% to 2.8% (with one exception) for neonatal primary cultures and from .1% to .45% for the cultures from older donors. Subjects in the present study were considerably older, and the number of cell generations in culture proportionally less: 9.3 to 19.8 for specimens not exposed to prior *in vivo* solar irradiation. Plating efficiencies were similarly reduced in these cultures. The lack of correlation between donor age and *in vitro* lifespan of cultured epidermal cells for the 5 subjects in this study is not surprising, as there are large variations in the culture lifespan of adult human diploid fibroblasts also, and many subjects are necessary to demonstrate the statistical relationship for this cell type [7,10,16]. These findings suggest that cultured epidermal cells are indeed comparable to cultured fibroblasts and amenable to the study of gerontologic problems.

Chronic sun exposure does accelerate aging in at least one regard. The actinically damaged kerotinocytes in this study had a uniformly decreased *in vitro* lifespan, compared to their nonexposed controls. The clinically "oldest," most severely damaged skin, that of an 80 yr old, yielded fewer than 40% the number of generations for his own nonexposed skin, while the sun-exposed but less actinically damaged skin of the middle-aged subjects yielded from 78% to 95% as many generations as the control cultures. This difference in generation number cannot be attributed to the smaller number of colonies or of total cells in the cultures obtained from nonexposed skin, as rate of keratinocyte colony growth is indépendent of colony density until the colonies are confluent in the dish, a condition not encountered in these experiments (James Rheinwald, personal communication). The uniformity of these 5 subjects with respect to complexion and intensity of past sun exposure greatly strengthens the conclusion that the magnitude of this disparity is indeed related to age and cumulative sun exposure as graphed in Fig 3 and not simply an artifact of volunteer selection in this small group.

An unexpected finding is the greatly increased plating efficiency of chronically sun-exposed keratinocytes. These cells were obtained by definition from the lateral, as opposed to the medial, aspect of the arm, but it is difficult to attribute this altered behavior in culture to innate differences between skin specimens from these adjacent sites. Plating efficiency in this system is the number of nonterminally differentiated keratinocytes in the basal layer of the epidermis which form visible colonies in culture, divided by total number of cells plated. This latter number includes total basal layer keratinocytes (the "true" or desired denominator) plus melanocytes, differentiated keratinocytes in the suprabasilar layers, dermal fibroblasts, endothelial cells, mast cells, and histiocytes. Chronic sun exposure may influence the thickness of the epidermis, the contour of the dermo-epidermal junction and probably to a lesser degree, the number of other cell types in a representative cross section of skin. However, neither previous histologic studies [2,3,17] nor examination of representative paired biopsies in this study (Fig 1) suggest the overall 10–30 fold decrease in epidermal and dermal cellular elements in sun-exposed as compared to nonexposed skin that would be necessary to explain this discrepancy anatomically. Rather, it seems that a much greater proportion of those nonterminally differentiated keratinocytes subjected to repeated *in vivo* solar irradiation are capable of continued cell division in culture, as compared to controls.

If repeated sun exposure damages occasional replicating keratinocytes in the basal layer, the skin may compensate by recruiting a larger proportion of basal cells into the dividing pool and proportionately decreasing the pool of resting "Go" cells [18]. Since actively dividing cells might be expected to adapt more readily to culture, such a phenomenon would explain the increased plating efficiency of keratinocytes from sun-exposed areas. Furthermore, increased proliferative demands *in vivo* resulting from selective cell destruction might reduce the reserve capacity of surviving undamaged keratinocytes to divide *in vitro*. Alternatively, exposed keratinocytes may experience sublethal actinic damage which is ultimately expressed as shortened culture lifespan.

This increased proliferative capacity of chronically sun-exposed epidermis is interesting in two regards. First, most of the clinically apparent actinic changes in skin are proliferative lesions involving one or more cell types: seborrheic keratoses, lentigenes, cherry angiomata, and telangiectasia (2). Second, repeated exposure to a carcinogen results in a persistently enlarged proliferative compartment in the hamster cheek pouch epidermis *in vivo* which antedates other evidence of malignant transformation [19] and in an increased plating efficiency *in vitro* for human diploid fibroblasts which are subsequently capable of tumor formation in mice [20]. Hence the finding of an increased proliferative pool of keratinocytes in biopsy specimens from chronically sun-exposed skin is consistent with the known carcinogenic effect of actinic irradiation on human skin [21], and measurement of relative plating efficiency for exposed and nonexposed keratinocytes in primary culture may provide a quantitative index of actinic damage or malignant potential.

Other investigators have already alluded to the need to avoid sun-exposed tissue when attempting to study chronologic aging changes [16], and the data presented here certainly substantiate this caveat.

Studies of the keratinocyte colonies were limited by their very poor growth potential. However, epidermal growth factor [22] and cholera toxin [23] both greatly increase the culture lifespan of human keratinocytes and should facilitate biochemical analyses of colonies from older donors in the future.

Finally, it is apparent that while chronically sun-exposed keratinocytes appear older than nonexposed keratinocytes of the same individual by virtue of a shortened culture lifespan, they are not identical to chronologically older cells. Further experiments will be necessary to determine how closely solar irradiation simulates the aging process.

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